

Polyomavirus early-late switch is not regulated at the level of transcription initiation and is associated with changes in RNA processing

(gene regulation/RNA splicing/polyadenylation/3'-end processing/RNA stability)

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ABSTRACT Polyoma gene expression is temporally regulated during productive infection of mouse cells. Early genes are expressed throughout the viral life cycle, but late mRNAs are not detected until after the onset of DNA replication. At late times, late-strand transcripts represent the great majority of viral-specific RNA in the cell. To learn more about the mechanism by which the early-late switch is regulated, we have carried out a detailed analysis of polyomavirus transcription in mouse NIH 3T6 cells. Nuclei were isolated from cells infected for 6, 12, 18, or 24 hr, and run-on assays were performed. The resulting RNAs were then hybridized to a number of immobilized early- and late-strand-specific probes, which represent the entire polyoma genome. Results indicate that the late promoter is always on, even in the absence of DNA replication. Even though the early-late switch is characterized by a >300-fold difference in the ratio of steady-state early- and late-strand RNAs, there is only a 2-fold effect at the level of transcription initiation. Furthermore, the efficiency of termination for late transcripts is very high at early times during infection (>90%) but drops drastically at late times (<40%). In other experiments, we have found an increase in splicing efficiency of late pre-mRNA molecules that parallels the decrease in termination efficiency. These results, taken together with other studies from our laboratory, have led us to propose two possible models for the temporal control of polyomavirus late gene expression.

Polyoma is a small, double-stranded, circular DNA virus whose genome contains two transcription units (early and late) and an intergenic control region (Fig. 1). The early transcription unit produces three differentially spliced mRNAs, which encode the three viral tumor antigens (small T, middle T, and large T). Three viral capsid proteins (VP1, VP2, and VP3) are encoded by three spliced messages from the late transcription unit. Viral gene expression proceeds in a well-defined, temporally regulated manner. Early genes are expressed throughout the virus life cycle; however, late messages and proteins accumulate only after viral DNA replication has begun (2–5).

The switch from early- to late-phase gene expression has been thought to occur as a result of large T antigen binding to high-affinity sites near the origin of replication leading to down-regulation of the early promoter (6) as well as by trans-activation of the late promoter by viral early proteins (7). Late promoter activation at the level of transcription initiation could result from the induction of a required late transcription factor or by overcoming negative regulation by a repressor molecule(s). However, other mechanisms could account for the low levels of transcripts from the late transcription unit seen before DNA replication. RNA polymerase could be initiated at the late promoter but may

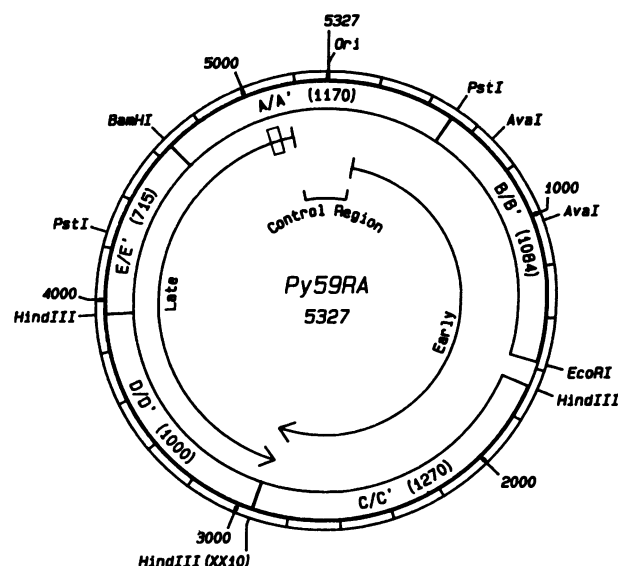


FIG. 1. Map of polyoma wild-type strain 59RA (1). Arrows represent the early and late transcription units. The box on the late transcription unit arrow represents the late leader. All known promoter elements and the origin of replication are contained within the intergenic control region. The blocks A/A'–E/E' represent genome fragments cloned into phage M13 cloning vectors to provide single-stranded probes for nuclear run-on transcription analysis. Probes A–E are complementary to early RNAs, while probes A'–E' are complementary to late RNAs. The numbers in brackets indicate fragment lengths.

encounter an elongation block preventing the production of full-length transcripts as shown for c-myc, c-myb, human immunodeficiency virus, β -globin, and *Drosophila melanogaster* hsp70 (8–12). Alternatively, transcripts could be unstable before DNA replication and then stabilized later in infection, similar to immunoglobulin heavy-chain expression (13).

The present study was undertaken to determine how the early-late switch occurs. The results show that even though the switch involves at least a 300-fold difference in RNA accumulation, there is only a 2-fold effect at the level of transcription initiation. The late promoter is on at all times during infection, even in the absence of DNA replication. However, late-strand termination efficiency near the poly(A) site changes from >90% before DNA replication to <40% after DNA replication. In addition, as infection proceeds, there is an increase in splicing efficiency of pre-mRNAs from the late transcription unit. Based on these observations, we present two models that may account for the temporal control of polyomavirus late gene expression.

MATERIALS AND METHODS

Plasmids and Phage Recombinants. All plasmids and phage recombinants were constructed by standard cloning techniques (14). The *Hind*III site in clone XX10 between the early and late coding regions at nucleotide (nt) 2921 (numbering as in ref. 2) was created by oligonucleotide mutagenesis and changes the sequence at nt 2905 5'-CAGTTTATTGAA-TAAACATTAA-3' to 5'-CAGTTTATTGAATAAAGCT-TAA-3' (G. Adami, personal communication). Both the early poly(A) site from nt 2915 to 2920 and the late poly(A) site from nt 2913 to 2908 are underlined. PGM7 and BLJ8 contain the *Bam*HI/*Pst* I fragment from nt 4632 to 488 (fragment A/A') cloned into the corresponding restriction sites in M13mp9 and M13mp8, respectively. PR-3 and 9-PR contain the *Pst* I/*Eco*RI fragment from nt 488 to 1560 (fragment B/B') cloned into the corresponding restriction sites in M13mp8 and M13mp9, respectively. RPHD7 and RPHD10 contain an *Hind*III/*Hind*III fragment from nt 1656 to 2921 (fragment C/C') cloned into the *Hind*III site of M13mp18 in both orientations. RPHD8 and RPHD11 contain the *Hind*III/*Hind*III fragment from nt 2921 to 3919 (fragment D/D') cloned into the *Hind*III site of M13mp18 in both orientations. RPHD9 and RPHD12 contain the *Hind*III/*Bam*HI fragment from nt 3919 to 4632 (fragment E/E') cloned into the corresponding restriction sites in M13mp18 and M13mp19, respectively. Single-stranded phage DNA from PGM7, PR-3, RPHD7, RPHD8, and RPHD9 will hybridize to early RNA; single-stranded phage DNA from BLJ8, 9-PR, RPHD10, RPHD11, and RPHD12 will hybridize to late RNA. All phage recombinants were verified by restriction enzyme mapping and dideoxy sequence analysis.

RNA probes were synthesized from plasmid pBSAva, which contains the *Ava* I/*Ava* I fragment of polyoma spanning the early splice sites cloned into pBS(+) (Stratagene) at the *Hinc*II site, and plasmid pBSVP1, which contains the polyoma *Hind*III/*Pst* I fragment spanning the VP1 splice cloned into the corresponding restriction sites in pBS(+).

Cells and Viruses. Propagation of viruses and cells were as described (15) except that NIH 3T6 cells were used.

RNA Isolation and RNase Protection Assay. Cytoplasmic and nuclear RNAs were isolated by a modification of the guanidine isothiocyanate method (14). The frozen cytoplasmic and nuclear fractions in guanidine described below were thawed and 1.5 ml of the samples were layered over a 0.5-ml cushion of 5.7 M CsCl and then spun at 40,000 rpm for 3 hr at 20°C in a Beckman TL100 ultracentrifuge using a TLS55 rotor. The resulting RNA pellet was resuspended in 300 μ l of 0.3 M NaOAc, extracted with phenol/chloroform (1:1), and stored as an EtOH precipitate at -80°C until use. Synthesis of probes with T3 RNA polymerase (Bethesda Research Laboratories) and RNase protection with RNase T2 (Bethesda Research Laboratories) were carried out as described (16). Resulting protected fragments were analyzed on 6% acrylamide/7 M urea sequencing gels.

Nuclear Run-On Transcription. Isolation of nuclei and nuclear run-on transcription was performed as described by Greenberg (17) with the following modifications. After lysing the cells and pelleting the nuclei, the supernatant was removed and added to 1.42 g of guanidine thiocyanate, frozen in liquid N₂, and stored at -80°C until cytoplasmic RNA was isolated as described above. The nuclear pellet was resuspended in 7 ml of Nonidet P-40 lysis buffer (16) and 3 ml was removed, added to guanidine, and reserved for nuclear RNA isolation as described above. The remaining nuclei were pelleted, frozen in liquid N₂, and stored at -80°C. Under the conditions used to prepare nuclei (10 mM Tris-HCl, pH 7.5/10 mM NaCl/3.0 mM MgCl₂/0.5% Nonidet P-40) it has been previously shown that few if any viral transcription complexes are extracted (18). After the transcription reaction

and RNA isolation by trichloroacetic acid precipitation and elution (16), the RNA was ethanol precipitated with 500 μ g of salmon sperm DNA and the pellet was dried and resuspended in 500 μ l of HESN [10 mM Hepes, pH 7.5/1 mM EDTA/0.2% sodium dodecyl sulfate (SDS)/0.1 M NaCl]. RNA was fragmented as described (17). To ensure that all RNA-RNA hybrids were denatured, the mixture was boiled for 10 min and quick chilled on ice. Two hundred microliters of 5 M NaCl was added to bring the NaCl concentration to 1 M. Hybridizations were in 10 mM Hepes/1 mM EDTA/1% SDS/1 M NaCl for 96 hr to the single-stranded DNA probes described above bound to GeneScreenPlus as recommended by the manufacturer. Filters were washed as described (17).

RESULTS

The Polyoma Early-Late Switch Involves Differential RNA Accumulation and Is Paralleled by a Change in Late pre-mRNA Splicing Efficiency. At 12 and 24 hr postinfection of mouse NIH 3T6 cells with a high multiplicity of wild-type polyomavirus, nuclear and cytoplasmic RNAs were isolated and the steady-state levels of polyoma E-RNA (transcripts from the early transcription unit) and L-RNA (transcripts from the late transcription unit) present were determined by an RNase protection assay. Fig. 2 shows that for nuclear RNA the ratio of E-RNA to L-RNA at 12 hr postinfection is \approx 4:1, while at 24 hr this value switches to 1:80. This 300-fold switch in RNA accumulation is even more dramatic when cytoplasmic RNA is examined (data not shown). When infections are carried out in the presence of cytosine β -D-arabinofuranoside (araC, a DNA synthesis inhibitor), E-RNA accumulates at normal rates, but L-RNA does not (Fig. 2). This is consistent with previous observations that the early/late RNA ratio changes dramatically during infection in a DNA replication-dependent fashion (3-5).

In addition to this difference in RNA accumulation, there is a parallel change in L-RNA splicing. Densitometric scanning of the RNase protection assay in Fig. 2 reveals that, as infection proceeds, the percentage of L-RNA with the mVP1 splice increases at least 3 times (from 16% to 53%); however, the percentage of spliced E-RNA does not change significantly (from 67% to 78%) (Fig. 2A). Identical results are obtained using cytoplasmic RNA (data not shown). In the presence of araC, the efficiency of mVP1 splicing remains at levels comparable to that seen 12 hr postinfection (Fig. 2B). Thus as infection proceeds, the efficiency of mVP1 splicing increases in a DNA replication-dependent manner, while the splicing efficiency of E-RNA remains unaffected.

Nuclear Run-On Analysis Can Distinguish Between Various Models for the Early-Late Switch. Possible models for the early-late switch can be divided in two types. First, if the switch is controlled at the level of transcription initiation, no RNA polymerase molecules should be transcribing the late strand before DNA replication. After the onset of DNA replication there should be a dramatic activation of the late promoter. In the second model, control at the level of elongation and/or RNA stability predicts that polymerases are initiated on the late strand before the onset of DNA replication. In such a model, an asymmetric distribution of polymerases would indicate a block to elongation (a pause and/or termination event) or differential elongation rates. The distinction between these models can be investigated by nuclear run-on transcription analysis.

Nuclei from polyoma-infected NIH 3T6 cells were isolated 6, 12, 18, and 24 hr after infection and used for nuclear run-on transcription as described in *Materials and Methods*. Since the early and late transcription units are read from opposite strands, labeled transcripts were hybridized to a number of single-stranded DNA probes to distinguish between transcription from the early transcription unit (E-strand) and the late transcription unit (L-strand). The results of these anal-

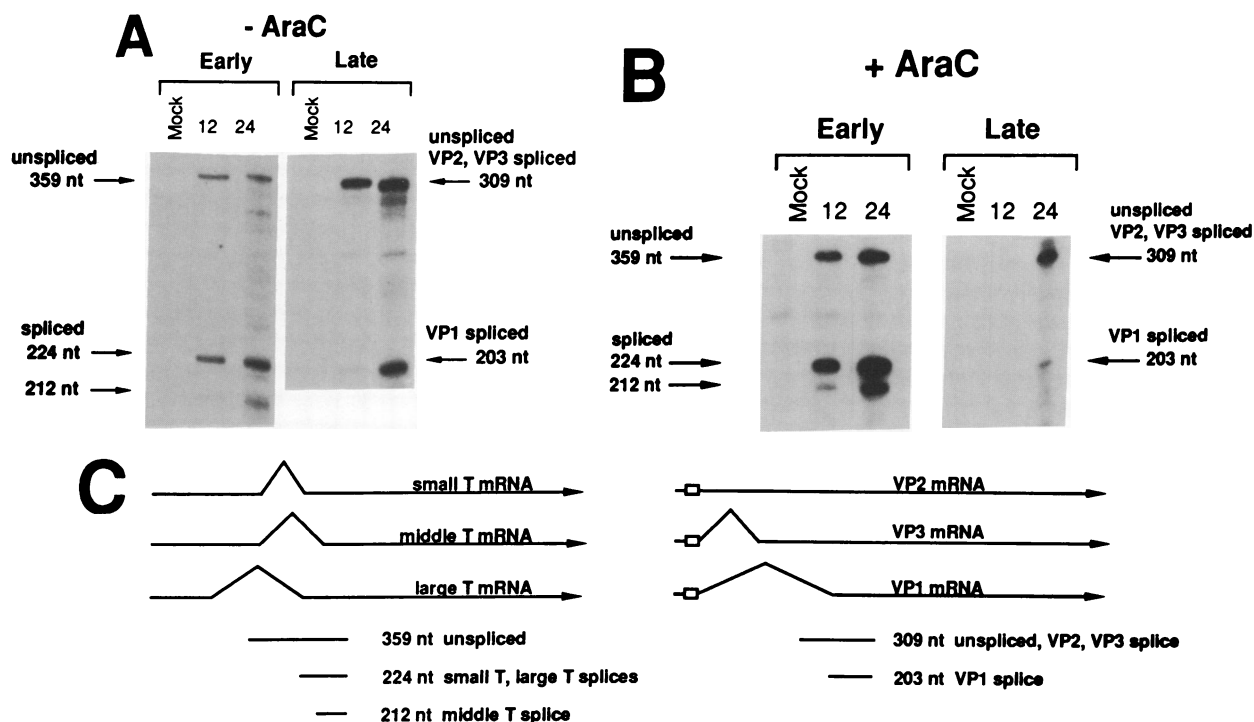


FIG. 2. RNA probe analysis of polyoma early and late transcripts. Nuclear RNA was isolated 12 and 24 hr postinfection from NIH 3T6 cells grown in the absence or presence of araC (20 μ g/ml) and subjected to RNA probe analysis. The resulting autoradiograms were scanned with a Bio-Rad 620 densitometer and the values obtained were corrected for fragment length. (A) RNA accumulation and splicing efficiency in the absence of araC. The following amounts of nuclear RNA were used: 12 hr early, 100 ng; 12 hr late, 500 ng; 24 hr early, 500 ng; 24 hr late, 10 ng. (B) RNA accumulation and splicing efficiency in the presence of araC. Seven hundred nanograms of nuclear RNA was used in all samples. (C) Schematic representation of the probes used and protected fragments obtained.

yses are presented in Fig. 3. Quantitation by scanning densitometry is presented in Table 1. This time course has been repeated 16 times with two cell lines and four different viral stocks. In all cases, the results were the same as those discussed below.

The Early Promoter Is Not Significantly Down-Regulated at the Level of Transcription Initiation at Late Times in Infection. For E-strand transcription, at all times during infection most of the polymerase molecules are found in the early coding region (Fig. 3). There is a large increase in signal between 6 and 12 hr, presumably due to DNA replication. This is apparent by examining the exposure times mentioned in the legend to Fig. 3. By comparing the signal from the L-strand to that of the E-strand, the early promoter may be down-regulated 2-fold 24 hr postinfection as has been suggested (6) (Table 1). However, these experiments cannot distinguish between early promoter down-regulation and late promoter activation. The possible significance of the unexpectedly low signal with signal observed with probe C will be explored further in the Discussion.

The Late Promoter Is Active Before the Onset of DNA Replication. The transcription pattern from the L-strand is inconsistent with current ideas of late promoter activation. RNA polymerase molecules transcribing the L-strand can be detected as early as 6 hr postinfection (Fig. 3). Even at 6 and 12 hr postinfection, the density of polymerases on the L-strand is higher than that on the E-strand (Fig. 3 and Table 1). This contrasts sharply with steady-state RNA levels, which show that E-RNA is 4 times more abundant than L-RNA at 12 hr postinfection. In addition, the relatively strong signal on A' and the low signal in E' at 6 and 12 hr indicates a high density of polymerase molecules at the 5' end of the late transcription unit, suggesting that L-strand transcription may be controlled partly by an elongation block before DNA replication. Curiously, this is not observed when

identical experiments are performed using another cell line, NIH 3T3 (data not shown).

At 18 and 24 hr postinfection, even though the ratio of steady-state E-RNA to L-RNA switches to 1:80 (a 300-fold change), nuclear run-on transcription assays indicate that the late promoter is activated only 2-fold relative to the early promoter (Fig. 3 and Table 1). As mentioned above, these experiments do not distinguish between down-regulation of the early promoter or activation of the late promoter. Regardless of which is true, this cannot account for the large differential accumulation of mRNAs, indicating that post-transcriptional events are involved. This conclusion is further supported by nuclear run-on analyses of infections carried out in the presence of araC (Fig. 3). The polymerase density profile for late-strand transcription at 24 hr postinfection under these conditions is identical to that seen 6 and 12 hr after infection without araC.

The signal observed with probe B' is somewhat confusing. At 6 hr postinfection, the signal observed is much higher than expected. This result is very reproducible. We have established that the single-stranded probes are in excess by making serial dilutions of the labeled transcripts and by treating the nuclei with RNase A as described by Schibler *et al.* (19) (data not shown), in each case obtaining identical results to those presented here. Using synthetic RNA probes made from a plasmid containing the entire polyoma genome, we have found that the results observed are not due to aberrant hybridization patterns (data not shown). Transcription carried out in the presence of novobiocin, an inhibitor of transcription initiation but not elongation (20), shows that the signals obtained are not due to polymerase molecules initiating *in vitro* (data not shown) and the addition of α -amanitin to 2 μ g/ml inhibits at least 95% of the signal seen, indicating that the signals observed are from RNA polymerase II (21) (Fig. 3). Even if the signal from probe B' is not used to calculate the late/early ratio

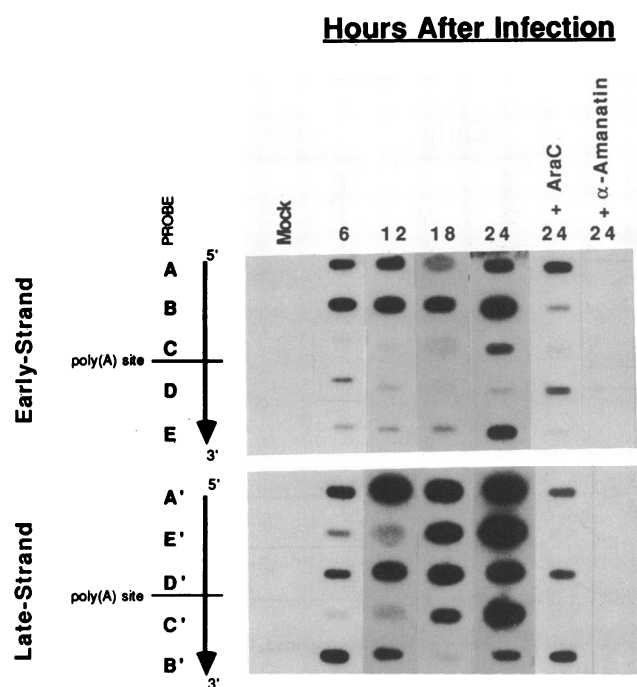


FIG. 3. Nuclear run-on analysis of polyoma-infected cells. NIH 3T6 cells were infected with polyoma and nuclei were isolated at the times indicated. Labeled transcripts were hybridized to five single-stranded probes specific for early-strand transcripts and five specific for late-strand transcripts (see Fig. 1). The early and late regions from each time point were exposed for the same amount of time as follows: Mock, 384 hr; 6 hr postinfection (hpi), 384 hr; 12 hpi, 20 hr; 18 hpi, 16 hr; 24 hpi, 20 hr; 24 hpi + araC, 384 hr; 24 hpi + α -amanatin, 16 hr. Signals from linear exposures were quantitated with a Bio-Rad 620 densitometer. All values were corrected for probe length and are presented in Table 1. araC indicates infections carried out in the presence of araC (20 μ g/ml) to inhibit DNA replication. α -Amanatin indicates transcription reactions carried out in the presence of α -amanatin (2 μ g/ml) to inhibit transcription by RNA polymerase II.

presented in Table 1, there is still only a 4-fold effect at the level of transcription initiation, not nearly enough to account for the 300-fold difference in RNA accumulation. The possibility remains that this signal is due to transcription from a previously unknown promoter. However, using RNA probe analysis, we have been unable to detect any polyoma-specific RNA 6 hr postinfection (data not shown).

The Termination Efficiency of Late Transcription Decreases as Infection Proceeds. The method of analysis used here can also be used to determine the efficiency of transcription termination between two adjacent fragments. It is apparent that the ratio of signals on D' and C' changes as infection proceeds. This change could be due to relieving termination/pausing within fragment D' or altering the efficiency of transcription termination associated with polyadenylation. [The late poly(A) site is at the junction of probe D' and probe C'.] In either case the efficiency of termination in fragment D' (%T_{D'}) can be calculated by the formula %T_{D'} = 2[signal in fragment D'/(signal in fragment D' + signal in fragment C')] - 100. Thus, if equal signals are obtained with both probes (50% in each), %T_{D'} = 0. Conversely, if all the signal is in fragment D', %T_{D'} = 100. After correcting for probe length (compare the bands in Fig. 3, probes D' and C'), it is apparent that termination for late-strand transcription at 6 and 12 hr postinfection is very efficient (>90%). In contrast, efficiency decreases to <40% at 18 and 24 hr. Termination is very efficient in infections carried out in the presence of araC, indicating that termination efficiency is linked to DNA replication.

Table 1. Quantitation of run-on data (arbitrary units)

Probe	Time postinfection, hr				
	6	12	18	24	24 + araC
Early-strand transcription					
A	6.8	8.6	2.2	9.7	5.9
B	12.1	21.7	10.1	48.9	2.3
C	0.7	0.3	1.3	2.9	0.9
D	1.7	0.8	0.3	0.9	2.8
E	4.6	1.4	1.4	18.7	1.7
Total	25.9	32.8	15.3	81.1	13.6
Late-strand transcription					
A'	6.2	72.8	17.3	88.1	3.6
E'	3.0	2.8	15.2	103.9	2.7
D'	4.0	20.1	7.0	30.4	4.0
C'	2.7	1.6	5.3	26.4	1.6
B'	19.1	6.3	0.9	5.4	14.3
Total	35.0	103.6	45.7	254.2	26.2
Late/early	1.4	3.2	3.0	3.1	1.9

Linear exposures from the data in Fig. 3 were scanned with a Bio-Rad 620 densitometer. The numbers shown were corrected for probe length. The numbers for early and late transcription from each time point were taken from the same exposure. The late/early ratio was calculated by dividing the total late signal by the total early signal.

However, at later times if termination becomes less efficient, the signal from probe B' is lower than expected by a factor of ≈ 5 . If polymerases are terminating transcription inefficiently late during infection, this signal should get stronger at the same rate as the signal from probe C'. As mentioned earlier, this result cannot be due to poor hybridization to this probe. One explanation for this distribution is that the elongation rate of polymerases through the region encompassed by probe B' is faster than the rest of the genome. Alternatively, a large proportion of the transcription complexes initiated at the late promoter may be terminating within this region.

DISCUSSION

During polyoma infection of permissive mouse cells there is a dramatic temporally regulated switch from E-strand-specific to mostly L-strand-specific gene expression (>300-fold change). This switch has been thought to be controlled at the level of transcription initiation via autoregulation of the early promoter and trans-activation of the late promoter after the onset of DNA replication (2-7). However, these processes may also be controlled at the level of transcription chain elongation and/or posttranscriptional processing. We have shown here that this temporally regulated switch is not controlled mainly at the level of initiation because initiated polymerase molecules can be found on both the early and late strands throughout infection. Furthermore, the ratio of polymerase molecules on the E-strand and L-strand changes only 2-fold as infection proceeds. Hence, gene expression from the late transcription unit and possibly from the early transcription unit must be controlled at the level of elongation and/or RNA stability.

Our approach to the analysis of the results obtained from nuclear run-on transcription has been to focus mainly on signals that change as infection proceeds. Throughout infection the transcription pattern from the E-strand changes remarkably little. Curiously, though, the signal from probe E at 24 hr is always much higher than expected. Whether this is the result of transcription from a minor promoter or represents polymerases stalled in this region awaits further experimentation. In addition, the signal from probe C is always much lower than expected. This is not due to poor hybridization efficiency to probe C because dilutions of the RNA transcripts give identical results. Experiments testing

the hybridization efficiency of all of the probes used have shown that probe C efficiently binds RNA. It is of interest to note that there is a minor polyadenylation site near the junction of probes B and C (22). In some transformed cells, this site is used very efficiently (22). It is possible that a large proportion of the transcripts from the early promoter are terminated at this site but are less stable than full-length transcripts.

L-strand gene expression undergoes striking changes during infection. We have shown here that even though L-mRNAs do not accumulate before DNA replication, initiated RNA polymerase II molecules are present on the L-strand at all times. In 3T6 cells, there is an apparent transcriptional block at the 5' end of the late transcription unit, which is removed as infection proceeds in a DNA replication-dependent manner. In addition, at early times (before DNA replication) a majority of the transcription complexes that bypass the block terminate (or pause) at or near the late poly(A) site. This could be due to a pause or termination signal located between the *Hind*III site at nt 3919 and the poly(A) site at nt 2913 or to termination associated with 3'-end processing. As infection proceeds, polymerase molecules traverse this region more and more efficiently. This change in efficiency may be controlled in several ways. A limiting factor(s) required for pausing/termination may be titrated out as DNA replication proceeds, or viral infection may induce a factor that acts as a negative regulator of this event. Alternatively, the transcription complex initiated at the late promoter may be modified in such a way that it no longer recognizes the pause/termination signal efficiently. All of these mechanisms would allow polyoma to produce giant primary transcripts at late times during infection, as has been observed (23). The formal possibility remains that the *in vitro* nuclear run-on patterns do not accurately reflect *in vivo* transcription rates; however, Falck-Pedersen *et al.* (24) have shown that short *in vivo* labeling gives results in complete agreement with run-ons from isolated nuclei.

Based on these observations, we suggest two models to account for the regulation of L-strand gene expression (leading to the early-late switch) in polyomavirus. In the first, a termination event near the poly(A) site prevents transcription complexes initiating at the late promoter from transcribing the signals required for polyadenylation. These transcripts are unstable and are rapidly degraded. As infection proceeds, replication commences and this termination event is alleviated by one of the mechanisms mentioned above, allowing the normal processing of L-RNA molecules. This model is consistent with the run-on analyses presented here but does not address other aspects of the early-late switch.

The second model, the one we favor, takes into account the data on the efficiency of mVP1 splicing presented above. With the single exception of the relatively low signal on probe B' at late times (Fig. 3), this model can account for all of our results on late-strand transcription and pre-mRNA splicing. It has been suggested (25) that termination efficiency and L-RNA accumulation are linked. In the second model, we propose that these three processes (termination, splicing, and accumulation) are coupled due to the involvement of the polyoma "late leader" exon. The late leader is a 57-base exon encoded only once in the viral genome but found in multiple tandem copies on polyoma late mRNAs (26). These repeats are generated by leader-to-leader splicing of giant primary transcripts produced as a result of inefficient termination (27). Recent studies on L-RNA splicing in our laboratory have indicated that before efficient mVP1 splicing is possible, leader-to-leader splicing must occur. If leader-to-leader splicing is blocked by exon truncation or by a variety of splice site mutations, the resulting L-RNA is highly unstable and mVP1 splicing is inefficient (28, 29). This is consistent with the observations of Kern *et al.* (30), who proposed that a

reiterated leader sequence was required for the stabilization of L-RNA transcripts. Thus, in our second model for the regulation of late gene expression, before DNA replication 3' processing for L-strand transcription is efficient, allowing for the production of primary transcripts containing only one late leader, making leader-to-leader splicing impossible. These pre-mRNA molecules cannot be spliced efficiently, and most are degraded in the nucleus. As infection proceeds, 3'-end processing becomes less efficient, allowing RNA polymerase II to make multiple circuits of the genome. These giant transcripts contain two or more late leader exons, which can be spliced together, thus allowing for efficient message body splicing and the dramatic accumulation of stable L-mRNAs after DNA replication.

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